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54 Method for the expression of heterologous genes in the yeast Pichia pastoris, expression vectors and transformed microorganisms.

57 The present invention provides a highly efficient method for the expression of heterologous genes, using as the host an auxotrophic his3 mutant of strain BKM-90 of Pichia pastoris and as recombinant DNA for the transformation thereof an integrative vector which contains a functional selection marker of Saccharomyces cerevisiae, the promotor of the alcohol oxidase gene (AOX₁) of the yeast itself, as the transcription terminator that of glyceraldehyde 3-phosphate dehydrogenase (GAP) of S. cerevisiae, or that which is carried by the heterologous gene to be expressed, and another sequence homologous to the genome of Pichia pastoris which serves for the integration thereof. The expression vectors used further contain a heterologous gene bound to the signal peptide of the sucrose invertase gene (SUC2) of S. cerevisiae. This method guarantees that the polypeptide products are recoverable under controllable conditions and with high yields. The strains according to the present invention can be used for the large-scale production of pharmaceutical products or of enzymes of industrial interest.

METHOD FOR THE EXPRESSION OF HETEROLOGOUS GENES IN THE YEAST PICHIA PASTORIS, EXPRESSION VECTORS AND TRANSFORMED MICROORGANISMS

The present invention is in the field of biotechnology and relates to recombinant DNA techniques, in particular to a new method for the efficient expression of heterologous genes in recombinant methylotrophic yeasts of the genus *Pichia*.

Within this field, an important object has been to develop host-vector systems useful in obtaining desired gene products, the great majority of which cannot be obtained in significant quantities from their natural sources. Numerous prokaryotic and eukaryotic expression systems have been described and used successfully to produce heterologous proteins with high yields.

10 Within this field, an important object has been to develop host-vector systems useful in obtaining desired gene products, the great majority of which cannot be obtained in significant quantities from their natural sources. Numerous prokaryotic and eukaryotic expression systems have been described and used successfully to produce heterologous proteins with high yields.

The cellular organism most exploited for these purposes has been the bacterium *E. coli*. This is to a large extent due to the knowledge which exists on its gene regulation and to the high feasibility of using it on a large scale.

Nevertheless, *E. coli* is not the most suitable host for the expression of any heterologous product, especially for proteins of pharmaceutical or dietary interest, since this organism produces toxic and pyrogenic components in its cell wall. Moreover, the mechanisms of transcription, translation and posttranslational processing differ from those of eukaryotic organisms, and hence proteins of eukaryotic origin produced in *E. coli* may differ from the normal gene products and lack the required biological activity. On the other hand, these products are usually insoluble and form protein aggregates that can be solubilised only by chemical modification, which may lead to a considerable decrease in their specific activity or in an increase in antigenic capacity of the product obtained. Another disadvantage of this host is its limited capacity for excretion of the proteins into the culture medium (E. Baron, "E. coli: an Old Friend but New Industrial Microbe", pp. 27-37; J. Davies, "Retrospect on Heterologous Gene Expression", pp. 17-26. Fifth International Symposium on the Genetics of Industrial Microorganisms, 1986. M. Alacevic, D. Hranueli, Z. Toman, eds.)

25 The ability to produce heterologous proteins in eukaryotic systems such as yeasts has certain advantages in relation to systems of the prokaryotic type. Among these advantages may be mentioned the ability to grow to high densities and therefore to adapt their cultures to continuous systems (US patent No. 4414329, assigned to Philips Petroleum Co.). Moreover yeasts may excrete the proteins into the culture medium in considerably higher quantities in comparison with *E. coli*, and the culture media used for yeast fermentation are generally more economical than those which are used in bacteria (Y. Lemoine, "Heterologous Expression in Yeast", 8th International Biotechnology Symposium, Paris, July 17-22, 1988).

30 Furthermore, these systems can perform posttranslational modifications such as glycosylation which is absent in bacterial systems (W. Fiers, "Engineering Maximal Expression of Heterologous Genes in Microorganisms", 8th International Biotechnology Symposium, Paris, July 17-22, 1988) and moreover may exhibit certain preferences for the codons used by cells of higher organisms, which promotes greater expression of genes from mammals (S.M. Kigsman et al. "Heterologous Gene Expression in *Saccharomyces cerevisiae*", Biotechnology & Genetic Engineering Reviews, Vol. 3, Ed. G.E. Russell)

35 In recent years methylotrophic yeasts, and among them in particular *Pichia pastoris*, have been used successfully for the cloning and expression of heterologous genes (Australian patent application No. 581107 by Philips Petroleum Co.), which makes it an attractive host for a number of advantageous characteristics over the one traditionally used for these purposes, *Saccharomyces cerevisiae*. The advantages comprise the ability to grow to very high cell densities and to use as the substrate for induction of the cloned genes, a cheap carbon source obtained as a waste material, as is the case with methanol. On the other hand, the system of gene expression of *Pichia* is more highly regulable compared with the expression systems described for *Saccharomyces*, which is an advantage for obtaining products which may have harmful effects in the hosts which produce them.

40 European patent application No. 183070 by Philips Petroleum describes a method for the transformation of a mutant of the yeast *Pichia pastoris* which is auxotrophic for the enzyme histidinol dehydrogenase, using as a selection marker the HIS4 gene of *Saccharomyces cerevisiae* and the HIS4 of the actual yeast *Pichia pastoris*, for which different transformation vectors of the replicative and integrative type were used.

45 The present invention provides, and relates to, a method for the expression of heterologous genes which uses as the host a new auxotrophic mutant strain of strain BKM-90 of *Pichia pastoris* (BKM, Moscow, USSR), which was called MP-36. This mutant is defective in the enzyme imidazole glycerophosphate dehydratase (IGP dehydratase) of the biosynthetic pathway of histidine and was obtained by mutagenesis with N-methyl-N-nitro-nitroso-guanidine (NTG) and with enrichment using nystatin.

A novel feature of the method is the use of the HIS3 gene of *S. cerevisiae* as a selection marker in the

new mutant strain of the genus *Pichia*.

This mutant was found to display high stability (frequency of reversion 10^{-8}), as well as to be capable of being transformed with high efficiency, which even surpasses the results expected for vectors of the integrative type such as those which are used in the present invention.

5 The vector constructed in order to accomplish selection of the mutant MP-36 is pHIS-85 (Fig. 1). This vector contains the HIS3 gene of *S. cerevisiae* which serves as a selection marker and also contains a chromosomal DNA fragment of *Pichia pastoris* which serves for integration.

This vector is constructed in order to check that the HIS3 gene of *S. cerevisiae* can complement a his⁻ mutation of *Pichia pastoris* and that the mutant MP-36 of said yeast can be transformed under these 10 conditions in a highly efficient manner.

Another novel feature of this method is the use of the integrative vector pPS-7 (Fig. 3) as a cloning vehicle for heterologous genes in *Pichia pastoris*. A characteristic of this vector is that it contains the HIS3 gene of *S. cerevisiae* as a selection marker. It also contains the promotor of the alcohol oxidase gene of *Pichia pastoris* (AOX₁), followed by the signal peptide of the sucrose invertase gene (SUC2) of *S. cerevisiae*; as a transcription terminator, it contains the transcription terminator of glyceraldehyde 3-phosphate dehydrogenase (GAPt) of *S. cerevisiae*. Alternatively, the termination signal of the actual gene which it is desired to express can be used (vector pPS-7A, Fig. 2).

The present invention further provides, and relates to, the expression vectors pPEGF-L, pPS-7A, pPSA-3, pPPC316-3 and pRH-4, which are used in a transformation of the mutant MP-36 to obtain heterologous 20 proteins such as epidermal growth factor, invertase, alpha-amylase, bovine rennin and fungal rennin respectively.

More specifically, for the expression of a heterologous gene there was selected the gene coding for human epidermal growth factor (hEGF) which is obtained by synthesis (Fig. 6). This gene was inserted in the integrative vector pPS-7 between the signal peptide of SUC2 and GAPt (Fig. 4). The host MP-36 was 25 transformed with this vector. The clone MEGF-5, to which the present invention also relates and which, on being fermented under suitable conditions and induced with methanol, displays levels of expression over 2.5 g/l culture, was selected from the transformants.

The high levels of expression reached for this protein made it possible to develop an efficient production process resulting in an end product of high purity which is suitable for pharmaceutical 30 application.

For the expression of sucrose invertase there was used the transformation vector pPS-7A, which differs from pPS-7 in that it does not contain GAPt. The host MP-36 was transformed therewith and the clone MSUC-2, to which the present invention also relates, was selected from the transformants. The levels of enzyme expression reached were greater than 4 g/l, the efficiency of this method for the expression of 35 heterologous genes using its own transcription terminator being verified.

The recombinant strain obtained, MSUC-2, has the characteristic of retention of the enzyme in the periplasmic space, which makes it more practical for use in immobilised cell systems.

In the case of alpha-amylase, a transformation of the mutant MP-36 with the vector pPSA-3 resulted in the clone MPA 3625 which is capable of hypersecretion of a thermo-stable alpha-amylase enzyme in 40 quantities greater than 6 g/l culture. The present invention also relates to this clone.

The present invention further provides, and relates to, the recombinant strains:

- CLRB-4, able to produce bovine chymosin within a range of 1-2 g/l culture and resulting from a transformation of the mutant MP-36 with the vector pPPC316-3, and
- CLRH-1, able to produce fungal chymosin (mycorennin) with levels greater than 7 g/l under 45 conditions of semicontinuous culture and resulting from transformation of the mutant MP-36 with the vector pRH-4.

The following strain deposits were made under the rules of the Budapest Treaty with the Centraalbureau voor Schimmelcultures (CBS), Baarn, the Netherlands, on October 22, 1990:

- CBS 454.90, *Pichia pastoris* CLRB-4 (pPPC316-3)
 50 CBS 453.90, *Pichia pastoris* CLRH-1 (pRH-4)
 CBS 452.90, *Pichia pastoris* MSUC-2 (pPS-7A)
 CBS 451.90, *Pichia pastoris* MPA 3625 (pPSA-3)
 CBS 449.90, *Pichia pastoris* MEGF-5 (pPEGF-L)

Practical examples of the present invention are described below.

55

EXAMPLE 1:

In order to accomplish mutagenesis of *Pichia*, cultures of yeast strain BKM-90 were inoculated in 100

ml YPG medium (yeast extract 1%, peptone 2%, glucose 2%), and incubated at 30 °C for 20 hours. 50 ml of culture were taken and centrifuged at 3000 r.p.m. for 5 min. The cells were washed twice with 0.1 M citrate buffer, pH 5 (sodium citrate 9.79 g, citric acid 3.2 g, and sufficient water for 500 ml) and resuspended in 50 ml of the same buffer. 10 ml of this suspension were taken and treated with a solution of 5 NTG to a final concentration of 50 g/l. The suspension was incubated in the presence of the mutagen for 30 min at 30 °C while standing.

The NTG was removed from the suspension by washing twice with distilled water. The cells were resuspended in 50 ml of YPG and transferred to an Erlenmeyer flask with 100 ml of this medium, being incubated at 30 °C with agitation for 48 h to allow expression of the mutants. Next 5 ml were extracted to 10 inoculate 100 ml of minimal G0 medium (1.32 g of (NH₄)₂HPO₄; 0.9 g of (NH₄)₂SO₄; 0.50 g of urea in one litre of redistilled water) which were used for enrichment with nystatin. After incubation of this culture until reaching 20 to 30% of the initial optical density (OD), it was treated with 25 ml of a nystatin solution. The treated culture was incubated at 30 °C for 30 min without agitation. Subsequently the nystatin was eliminated from the medium by washing the cell suspension twice with distilled water, and resuspending the 15 cells in a volume sufficient to obtain between 150 and 200 colonies per dish.

The colonies were grown in complete medium and replicated in minimal medium (G0) for the isolation of auxotrophic mutants. Then the latter were characterised with respect to their nutritional deficiency, using the plasmid pHis-85 which carries the HIS3 gene of *Saccharomyces cerevisiae* (Fig. 1).

Using this method, the mutant MP-36 of *Pichia pastoris* was isolated, which is defective in the 20 biosynthetic pathway of histidine, and in particular in the enzyme imidazole glycerophosphate dehydratase (IGP dehydratase).

EXAMPLE 2:

25 With the object of creating a vector for the expression of heterologous genes in the mutant MP-36, the procedure was as follows:

The vector plasmid pUC-18 was digested with the enzymes BamHI and Sall, being subsequently ligated to the BamHI fragment of the vector pPMC which contains the HIS3 auxotrophy marker of *S. cerevisiae*; in turn, the BamHI/Sall fragment of the plasmid pCAO-10, which includes the AOX₁ gene plus a 3'-flanking 30 fragment, was ligated. As a result of this triple reaction, the plasmid pHAX-1 was obtained (Fig. 2).

Then the plasmid obtained was digested with the enzymes EcoRI and SmaI and ligated to the EcoRI/PstI fragment of the plasmid pCAO-10, the 5'-flanking fragment of the AOX₁ promotor which is used to increase the frequency of recombination by that end. At the same time there was ligated the EcoRI/PstI fragment of the plasmid pAS-24, previously filled in with the Klenow fragment at the EcoRI end; this region 35 contains the link of the AOX₁ promotor to the coding region of the SUC2 gene. From this reaction was obtained the vector pPS-7A (Fig. 2).

A fragment containing GAPt was extracted from the plasmid pBG3, which contains the GAP operator of *S. cerevisiae*, by digestion with the enzymes Ncol/BamHI. This fragment was introduced into the plasmid pPS-7A, from which the structural gene of SUC2 was previously extracted by digestion with the enzymes 40 HindIII/BamHI. In this ligation was introduced an 48-mer oligonucleotide with sticky 5' HindIII and 3' Ncol ends (Fig. 5), which restored the signal peptide sequence of the SUC2 gene (spSUC2) which was lost in the digestion, and also restored the recognition site for the enzyme Ncol. The resulting vector plasmid was called pPS-7, and this contained the AOX₁ promotor followed by the signal peptide of SUC2, then GAPt with a Ncol cleavage site between the two in order to allow the insertion of the heterologous gene which it is 45 sought to express; further on the HIS3 gene of *S. cerevisiae* is found, and finally the 3'-terminal fragment of the AOX gene for integration in the yeast (Fig. 3).

EXAMPLE 3:

50 Given the amino acid sequence of human (h) EGF, chemical synthesis of the gene which codes for this protein of approximately 6.2 kDa was performed. This gene has been the subject of prior publication (Chiron Corp., US patent 4783412), as well as chemical synthesis thereof (Earth Chemical, French patent 2566799). By the method described (H. Coster, N.D. Sinha, J. Biernat and J. McManus; NAP, vol. 12, pp. 4539-4557, 1984) there was accomplished the synthesis of a gene of 174 base pairs which codes for hEGF (Fig. 6).

55 Then this gene was inserted in the plasmid pBR322 which had been digested with the restriction enzyme EcoRV and treated with phosphatase (Fig. 4). This ligation resulted in the plasmid pBEGF which was multiplied by transformation of the *E. coli* strain MC1061. Sufficient plasmid material for extraction of the fragment corresponding to the hEGF gene was purified from this strain. A fragment containing said gene

and having blunt ends was successfully extracted by consecutive digestion with the restriction enzymes XbaI, SI and EcoRV.

For expression thereof in yeast, this fragment was introduced into the plasmid pPS-7 (the construction of which is described in the previous example), which was previously digested with the enzymes NcoI, SI and finally treated with phosphatase. The plasmid resulting from this ligation was called pPEGF-L (Fig. 4). Herein the gene coding for hEGF is located after the SUC2 signal peptide, under control of the AOX₁ promotor and combined with the GAP terminator, while the 5' and 3' ends of AOX serve as segments for integration. The transformation in yeast was carried out with this plasmid, and in particular Pichia pastoris his3 mutant MP-36 was used as the host.

10 The transformed strain, which was called MEGF-5, is capable of producing between 2.5 and 3 g EGF per litre.

EXAMPLE 4:

15 For expression of the sucrose invertase gene, P. pastoris strain MP-36 was transformed with the plasmid pPS-7A (obtained in one of the intermediate stages of construction of the vector pPS-7, Fig. 2), which carries the SUC2 gene under control of the AOX₁ promotor, and which has as transcription terminator that of the actual SUC2 gene. A similar experiment was performed using honey as a culture medium (industrial medium), which contained 4% total reducing sugars.

20 In both cases a high productivity of the system was achieved, between 3.5 and 4 g of enzyme per litre culture medium being obtained, and also having as a distinguishing characteristic the retention of this enzyme in the periplasm of the transformed microorganism.

EXAMPLE 5:

25 For cloning of the bovine chymosin gene there was used as a source the construct pCB-125 designed for E. coli (Morales, 1989, Rev. Interferón y Biotecn 5(2)), an NcoI site being formed by the polymerase chain reaction (PCR) technique (R. Saiki, 1985, Science 230: 1350-1354) for subsequent manipulation of the gene, and for this purpose the following oligonucleotides were synthesised chemically:

30 5' of the gene 5'GTGCCATGGCTGAGATCACCAGGATCCCT 3'
3' of the gene 5'GTGTCAAGATCAGATGGCTTGGCCAGC 3'.

After amplification the fragment was digested with NcoI, XbaI and subcloned at these sites in the vector pSAO7 with origin of replication of E. coli, being transformed in strain MC1061 and the transformants being selected by hybridisation of colonies with the 5' oligonucleotide designed for the PCR, and the positive clones being verified by restriction analysis with the enzymes NcoI, XbaI and HindIII.

35 For subcloning of the bovine chymosin gene, the vector pPS7 was linearised with NcoI, and the palindromic ends were made blunt by the action of SI nuclease, and then phosphorylated. The fragment with the prochymosin gene was obtained from the vector RePSAO by digestion with NcoI, HindIII, and the palindromic ends were filled in, becoming blunt by the action of the Klenow enzyme. In this way it was prepared for introduction thereof in the vector pPS7, giving rise to the integration vector pPPC316-3 (Fig. 7). With this last vector, transformation of the mutant MP-36 was carried out, strain CLR-B-4 being obtained which produces chymosin levels greater than 1.5 g/l culture.

EXAMPLE 6:

45 For extraction of the gene which codes for aspartic protease of strain IFO 4578 + of Mucor pusillus, purification of the genome of said fungus was carried out. For this, the mycelium was grown in wheat bran extract and the fungal DNA was extracted and purified as reported in the literature (Cryer et al., Methods in Cell Biology, 1975, Vol. XII, pp. 39-44).

50 Then the PCR technique was carried out (polymerase chain reaction, R.K. Saiki et al., 1985, Science 230: 1350-1354), for which were used two oligonucleotides the sequences of which are shown in Fig. 8. After amplification and extraction of the gene of the inactive form aspartic proprotease of M. pusillus, this was cloned in Bluescript (G. Wahl, 1989, "Strategies," 2:17), having first been bound to a BamH linker, to obtain the resulting plasmid pRH-3 (Fig. 8).

55 In order to carry out cloning of the gene which codes for fungal aspartic protease, the integrative vector pPS7 was cleaved with NcoI, and treated with SI nuclease and then alkaline phosphatase (CIP) (Maniatis et al., 1982, Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Laboratory, USA). This site permitted introduction of the desired genetic information from the plasmid pRH-3, exactly between the

secretion signal of the SUC2 gene of S. cerevisiae and the transcription termination signal of the GAP gene of the Saccharomyces itself, this information remaining under the control of the AOX₁ gene promotor of Pichia pastoris (Fig. 9). This expression cassette was completed with the HIS3 gene of Saccharomyces which was used as a selection marker, and the 5' and 3'-flanking regions of the actual AOX gene which were used for integration. The resulting expression vector was called pRH-4.

Then transformation of Pichia pastoris his⁻ mutant MP36 was carried out with the expression vector pRH-4, and the microorganism resulting from this, called CLRH-1, was grown and induced for excretion of the enzyme under conditions of semicontinuous culture.

For fermentation thereof, the new recombinant strain of the genus Pichia, which produces aspartic protease, was inoculated in a 5-litre fermenter containing industrial medium (13.2 g ammonium phosphate, 9 g ammonium sulphate, 5 g urea and 84 ml molasses per litre of culture medium, which yield 3% total reducing material). The inoculum corresponds to an optical density of 0.5-1 at 600 nm. Fermentation conditions were maintained at an incubation temperature of 30 °C and a pH of the medium of 5.5. Induction of the system was carried out by using methanol, when the culture reached the early exponential phase of its growth, and from that moment on a constant flow of 2.5 ml/h was maintained.

Under these conditions the recombinant strain CLRH-1 reached excretion levels of the enzyme of the order of 1.5 g/l culture, which increased considerably up to approximately 7 g/l culture by successive replacement with fresh medium as each batch was completed, or by semicontinuous culture.

20 EXAMPLE 7:

The chromosomal DNA extracted from Bacillus licheniformis CIB-25 (CIGB's collection of strains) was completely digested with the endonuclease EcoRI, and the fragments having a size between 2 and 4 kb were isolated by low melting point agarose gel electrophoresis (LGT). These fragments were ligated to the 26 vector pBR322 digested with EcoRI and treated with alkaline phosphatase. With the resulting plasmid, E. coli strain MC1066 (F, D lacX74, hsr, hsm, rps 1, galU, galK, tripl C 9030F, leuB, pyrF::tn5) was transformed.

The identification of clones was carried out by activity on plates, by seeding the colonies in LB medium with 0.5% soluble starch and tetracycline at 15 µg/ml. The colonies were left to grow at 37 °C for 72 hours and then the plates were stained with metallic iodine vapours. The positive clones were those which 30 displayed formation of a clear halo around the colony. One of the selected clones was called pAB-24, and restriction analysis was carried out with endonucleases. The restriction map of the 3 kb EcoRI fragment which contains the alpha-amylase gene is shown in Fig. 10. The restriction pattern obtained in the central part of the 3 kb fragment coincides with the results of S.A. Ortlepp et al., 1983, Gene 23: 267-276 and G.L. Gray et al., 1986, J. Bacteriol. 168: 635-643.

After analysing the clones and verifying that the structural region coincided with those reported, the 35 region which codes for the gene was isolated with precision. For this purpose 2 synthetic oligonucleotides were designed to perform a polymerase chain reaction (PCR). These oligonucleotides were designed on the basis of the published alpha-amylase sequence of B. licheniformis (G.L. Gray et al., 1986, J. Bacteriol. 168: 635-643). The sequences of both oligonucleotides are shown in Fig. 11. The oligonucleotide corresponding 40 to the 5' region of the gene includes an Ncol site, and the one corresponding to the 3' region of the gene a Sall site. To carry out amplification of the isolated alpha-amylase gene, the method described by R.K. Saiki et al., 1988, Science 239: 487-491, was followed.

According to the published sequence and the design of the oligonucleotides, the polymerase chain 45 reaction product must yield a 1.4 kb fragment which comprises the mature protein and does not contain its signal sequence. Given that the fragment obtained as the PCR product coincides with the desired size, it is established that there is very high homology between the alpha-amylase gene of B. licheniformis as published and the one isolated in this study. The isolated gene was digested with the enzyme Ncol in order to be subcloned in the plasmid YIP 5, the plasmid pAMSS being formed (Fig. 12).

The plasmid pPS-7 was used as a cloning vector by digestion with the endonuclease Ncol, treatment 50 with SI exonuclease and dephosphorylation of the ends with alkaline phosphatase. In this vector was inserted the alpha-amylase gene obtained from the plasmid pAMSS by digestion with the enzymes Ncol and Sall and making both ends of the fragment blunt with SI exonuclease. In this way they were linked in the final cloning vector pPSA-3 (Fig. 12), which was used for transformation of the mutant MP-36.

The transformants obtained were analysed for their capacity to produce alpha-amylase, for which they 55 were grown on plates of medium G (Galzy and Slonimski, 1957, (Paris) C.R. Acad. Sci., 245: 2423) with 1% soluble starch and 0.5% methanol. After 3 days' incubation at 30 °C, the plates were stained with iodine vapours, a clear halo being observed around the yeasts which produce the enzyme, due to starch hydrolysis.

One of the transformed yeasts, MPA 3625, which have a positive result by the plate selection method, was isolated in order to carry out fermentation-induction studies. It was capable of secreting the enzyme alpha-amylase in active form into the culture medium in quantities greater than 6 g/l in a process of fermentation and induction with methanol.

5

SEQUENCE LISTING

10 SEQ ID NO:1
SEQUENCE TYPE: nucleotide
SEQUENCE LENGTH: 29 nucleotides
MOLECULE TYPE: DNA
15 GTGCCATGGC TGAGATCACC AGGATCCCT 29

20 SEQ ID NO:2
SEQUENCE TYPE: nucleotide
SEQUENCE LENGTH: 28 nucleotides
MOLECULE TYPE: DNA
25 GTGTCAAGAT CAGATGGCTT TGGCCAGC 28

30 SEQ ID NO:3
SEQUENCE TYPE: nucleotide
SEQUENCE LENGTH: 49 nucleotides
MOLECULE TYPE: DNA
35 AGCTTTCCCTT TTCCCTTTGG CTGGTTTGCG AGCCAAAATA TCTGCAGGC 49

40 SEQ ID NO:4
SEQUENCE TYPE: nucleotide
SEQUENCE LENGTH: 49 nucleotides
MOLECULE TYPE: DNA
45 CATGGCCTGC AGATATTTG GCTGCAAAAC CAGCCAAAAG GAAAAGGAA 49

45

50

55

SEQ ID NO:5

SEQUENCE TYPE: nucleotide with corresponding protein

SEQUENCE LENGTH: 174 base pairs

STRANDEDNESS: single

TOPOLOGY: linear

MOLECULE TYPE: genomic DNA

ORIGINAL SOURCE ORGANISM: human

IMMEDIATE EXPERIMENTAL SOURCE: nucleotide synthesis

PROPERTIES: human epidermal growth factor gene

TCTAG AAC TCA GAT AGT GAA TGC CCT TTG AGC CAC GAT GGA TAT 44
 15 Asn Ser Asp Ser Glu Cys Pro Leu Ser His Asp Gly Tyr
 1 5 10

TGC CTA CAT GAC GGT GTT TGT ATG TAT ATC GAG GCT TTA GAC 86
 20 Cys Leu His Asp Gly Val Cys Met Tyr Ile Glu Ala Leu Asp
 15 20 25

AAA TAC GCA TGC AAC TGC GTT GGT TAC ATC GGA GAA AGA 128
 25 Lys Tyr Ala Cys Asn Cys Val Val Gly Tyr Ile Gly Glu Arg
 30 35 40

TGT CAA TAC CGT GAC TTA AAA TGG TGG GAA TTA CGT TAA TGATATC 174
 30 Cys Gln Tyr Arg Asp Leu Lys Trp Trp Glu Leu Arg *
 45 50

SEQ ID NO:6

SEQUENCE TYPE: nucleotide

SEQUENCE LENGTH: 31 nucleotides

MOLECULE TYPE: DNA

TCTAGAACTC AGATAGTGAA TGCCCTTGAG 31
 40

SEQ ID NO:7

SEQUENCE TYPE: nucleotide

SEQUENCE LENGTH: 37 nucleotides

MOLECULE TYPE: DNA

CCACGATGGA TATTGCCTAC ATGACGGTGT TTGTATG 37
 50

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SEQ ID NO:8
SEQUENCE TYPE: nucleotide
5 SEQUENCE LENGTH: 38 nucleotides
MOLECULE TYPE: DNA
TATATCGAGG CTTTAGACAA ATACGCATGC AACTGCGT 38

10 SEQ ID NO:9
SEQUENCE TYPE: nucleotide
SEQUENCE LENGTH: 37 nucleotides
15 MOLECULE TYPE: DNA
TGTTGGTTAC ATCGGAGAAA GATGTCAATA CCGTGAC 37

20 SEQ ID NO:10
SEQUENCE TYPE: nucleotide
SEQUENCE LENGTH: 31 nucleotides
MOLECULE TYPE: DNA
25 TTAAAATGGT GGGATTACG TTAATGATAT C 31

30 SEQ ID NO:11
SEQUENCE TYPE: nucleotide
SEQUENCE LENGTH: 21 nucleotides
MOLECULE TYPE: DNA
35 GATATCATTAA ACGTAATTCC C 21

40 SEQ ID NO:12
SEQUENCE TYPE: nucleotide
SEQUENCE LENGTH: 38 nucleotides
MOLECULE TYPE: DNA
45 ACCATTTAA GTCACGGTAT TGACATCTT CTCCGATG 38

50 SEQ ID NO:13
SEQUENCE TYPE: nucleotide
SEQUENCE LENGTH: 38 nucleotides
MOLECULE TYPE: DNA
55 TAACCAACAA CGCAGTTGCA TGCGTATTTG TCTAAAGC 38

SEQ ID NO:14
 SEQUENCE TYPE: nucleotide
 5 SEQUENCE LENGTH: 37 nucleotides
 MOLECULE TYPE: DNA
 CTCGATATAC ATACAAACAC CGTCATGTAG GCAATAT 37

10 SEQ ID NO:15
 SEQUENCE TYPE: nucleotide
 SEQUENCE LENGTH: 40 nucleotides
 15 MOLECULE TYPE: DNA
 CCATCGTGGC TCAAAGGGCA TTCACTATCT GAGTTCTAGA 40

20 SEQ ID NO:16
 SEQUENCE TYPE: nucleotide
 SEQUENCE LENGTH: 30 nucleotides
 MOLECULE TYPE: DNA
 25 TCGTCGCCA GTATCCAAGC AATCTGATGC 30

30 SEQ ID NO:17
 SEQUENCE TYPE: nucleotide
 SEQUENCE LENGTH: 30 nucleotides
 MOLECULE TYPE: DNA
 35 TTAGTTGTTC TCGTATCCGG AAGCCAAAGG 30

40 SEQ ID NO:18
 SEQUENCE TYPE: nucleotide
 SEQUENCE LENGTH: 22 nucleotides
 MOLECULE TYPE: DNA
 45 GCCATGGCAA ATCTTAATGG GA 22

50 SEQ ID NO:19
 SEQUENCE TYPE: nucleotide
 SEQUENCE LENGTH: 27 nucleotides
 MOLECULE TYPE: DNA
 CGTCGACCTA TCTTTGAACA TAAATTG 27

55 Claims

1. Method for the expression of heterologous genes in the yeast *Pichia pastoris*, comprising the steps of preparing an expression vector which contains the heterologous gene to be expressed, transforming a

- P. pastoris host strain, growing a transformant which produces the expression product of the heterologous gene and optionally isolating the expression product formed, characterised in that the expression vector is derived from a plasmid containing an expression cassette which comprises the AOX₁ promotor of P. pastoris, the signal peptide sequence of SUC2 of Saccharomyces cerevisiae followed by a restriction site for insertion of the heterologous gene to be expressed, optionally the transcription terminator of the GAP gene of S. cerevisiae behind this restriction site, and the HIS3 selection marker of S. cerevisiae, said expression cassette being flanked by 5' and 3' sequences of the AOX gene of Pichia to allow integration in the Pichia genome, and that a his⁻ mutant of P. pastoris is used as the host strain.
2. Method according to claim 1, characterised in that the P. pastoris his⁻ auxotrophic mutant MP-36 which is defective in the enzyme imidazole glycerophosphate dehydratase is used as the host strain.
3. Method according to claim 1 or 2, characterised in that the expression vector is derived from the plasmid pPS-7 which contains the transcription terminator of the GAP gene of S. cerevisiae.
4. Method according to claim 3, characterised in that the expression product of the heterologous gene is selected from the group consisting of human epidermal growth factor, alpha-amylase, bovine rennin and mycorennin.
5. Method according to claim 1 or 2, characterised in that the expression vector is derived from the plasmid pPS-7A which does not contain the transcription terminator of the GAP gene of S. cerevisiae, the expression vector derived therefrom comprising a heterologous gene which contains its own transcription terminator.
6. Method according to claim 5, characterised in that the expression product of the heterologous gene is sucrose invertase.
7. Method for the expression of heterologous genes in the yeast Pichia pastoris, characterised in that it uses as a system of expression for heterologous genes the vector of integration in yeasts pPS-7 which includes the AOX₁ promotor of P. pastoris, the signal peptide of SUC2 of S. cerevisiae, as a transcription terminator that of the GAP gene of S. cerevisiae and the HIS3 gene of S. cerevisiae as a selection marker, which form an expression cassette which is flanked by 5' and 3' sequences of the AOX gene of Pichia for integration, and as a host an auxotrophic mutant specific to the enzyme imidazole glycerophosphate dehydratase, MP-36, capable of reaching high levels of expression of a heterologous gene which is located between the signal peptide and the terminator.
8. Method for the expression of heterologous genes in the yeast Pichia pastoris, according to claim 7, characterised in that the expression vector, being called pPS-7A, does not carry the GAP termination signal, using for these purposes the own terminator of the heterologous gene which is inserted in it for expression thereof.
9. Expression vector as defined in any of the claims 1-8, comprising the AOX₁ gene promotor of Pichia pastoris, as well as 5' and 3' sequences of this gene for integration thereof in the genome of the yeast, the signal peptide of SUC2 of S. cerevisiae as an excretion signal and the HIS3 gene of S. cerevisiae as a selection marker, where the heterologous gene to be expressed is located between the signal peptide and a transcription termination sequence.
10. Expression vectors pPEGF-L, pPSA-3, pPPC316-3 and pRH-4, according to claim 9, containing as heterologous genes those which code for human epidermal growth factor (hEGF), alpha-amylase, bovine rennin and mycorennin respectively, and carrying as the transcription terminator that of the GAP gene of S. cerevisiae.
11. Expression vector pPS-7A according to claim 9, containing as heterologous gene the gene which encodes sucrose invertase (SUC2) of S. cerevisiae and carrying as the transcription terminator that of its own gene.
12. Transformed microorganism as defined in any of the claims 1-8, which expresses high levels of a

heterologous protein and results from transformation of mutant MP-36 with an expression vector which carries the gene coding for said protein.

13. Transformed microorganisms MEGF-5, MSUC-2, MPA 3625, CLR-B-4 and CLR-H-1, according to claim
5 12, which express high levels of hEGF, periplasmic sucrose invertase, thermo-stable alpha-amylase,
bovine rennin and mycorennin, resulting from transformation with the vectors pPEGF-L, pPS-7A, pPSA-
3, pPPC316-3 and pRH-4, respectively.
14. Products resulting from the expression of heterologous genes as defined in any of the claims 1-13.

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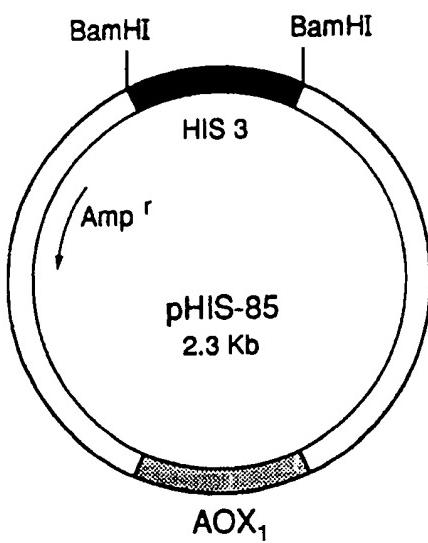


FIG. 1

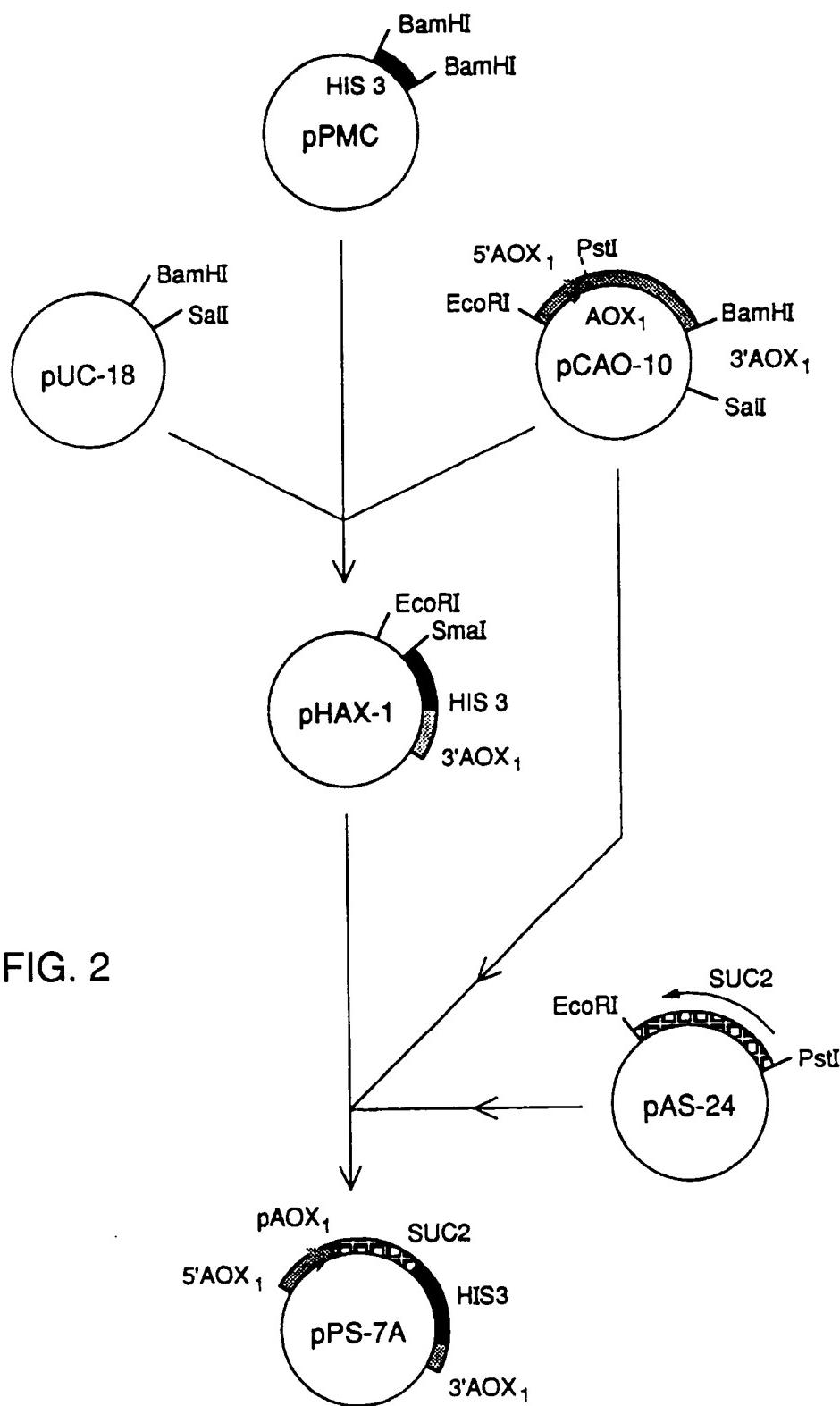


FIG. 2

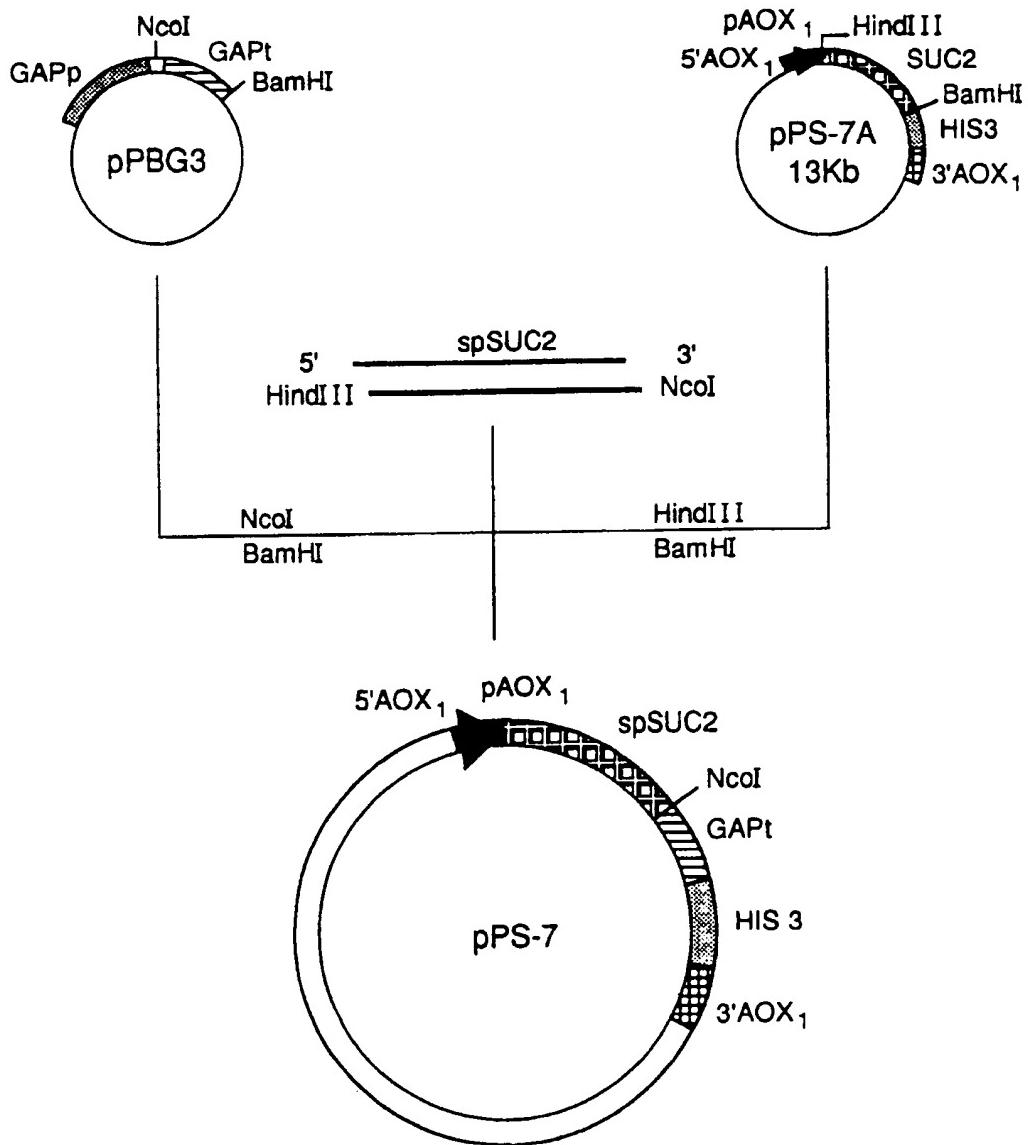


FIG. 3

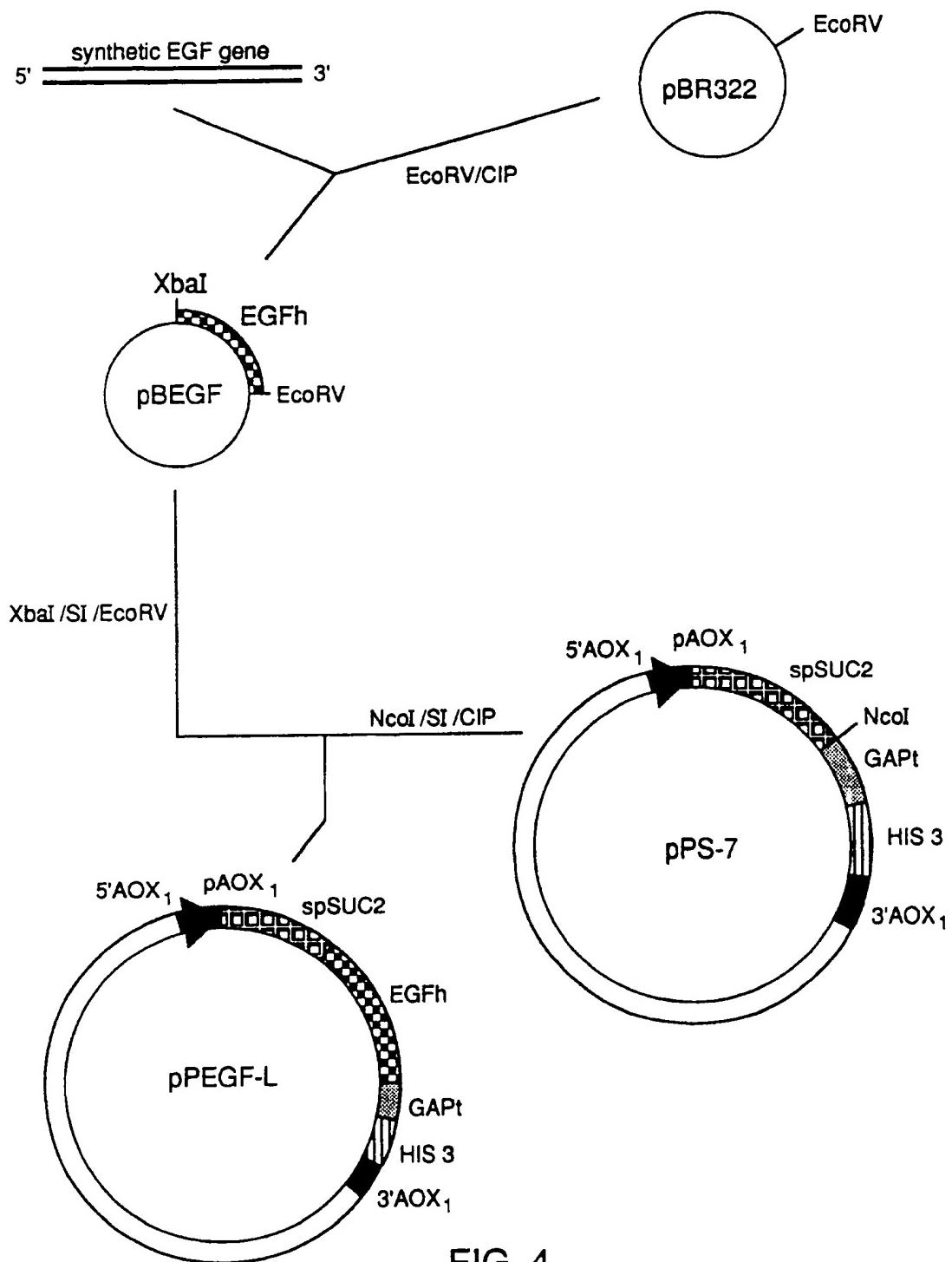


FIG. 4

Synthetic oligo which codes for the signal peptide of SUC2

AGC TTC CTT TTC CTT TTG GCT GGT TTT GCA GCC AAA ATA TCT GCAGGC
AG GAA AAG GAA AAC CGA CCA AAA CGT CGG TTT TAT AGA CGT CCG GTA C

FIG. 5

Nucleotide sequence of the gene coding for human EGF and oligonucleotides used in its synthesis.

TCTAGAACTCAGATAGTGAATGCCCTTGAGCCACGATGGATATTGCCTACATGACGGTG 60
TTTGTATGTATATGCAGGCTTAGACAAATACGCATGCAACTGCGTTGGTTACATCG 120
GAGAAAAGATGTCAATAACCGTGACTTAAATGGTGGGAATTACGTTAATGATATC 174

Series of oligonucleotides for the synthesis.

5' TCTAGAACTCAGATAGTGAATGCCCTTAGG
CCACGATGGATATTGCCTACATGACGGTGTGTATG
TATATCGAGGCTTAGACAAATACGCATGCAACTGCGT
TGTTGGTTACATCGGAGAAAGATGTCAATACCGTGAC
TTAAAATGGTGGGAATTACGTTAATGATATC
GATATCATTAACGTAATTCCC
ACCATTAAAGTCACGGTATTGACATCTTCTCCGATG
TAACCAACAACGCAGTTGCATGCGTATTGTCTAAAGC
CTCGATATAACATAACAAACACCGTCATGTAGGCAATAT
CCATCGTGGCTCAAAGGGCATTCACATTCTGAGTTCTAGA
3'

FIG. 6

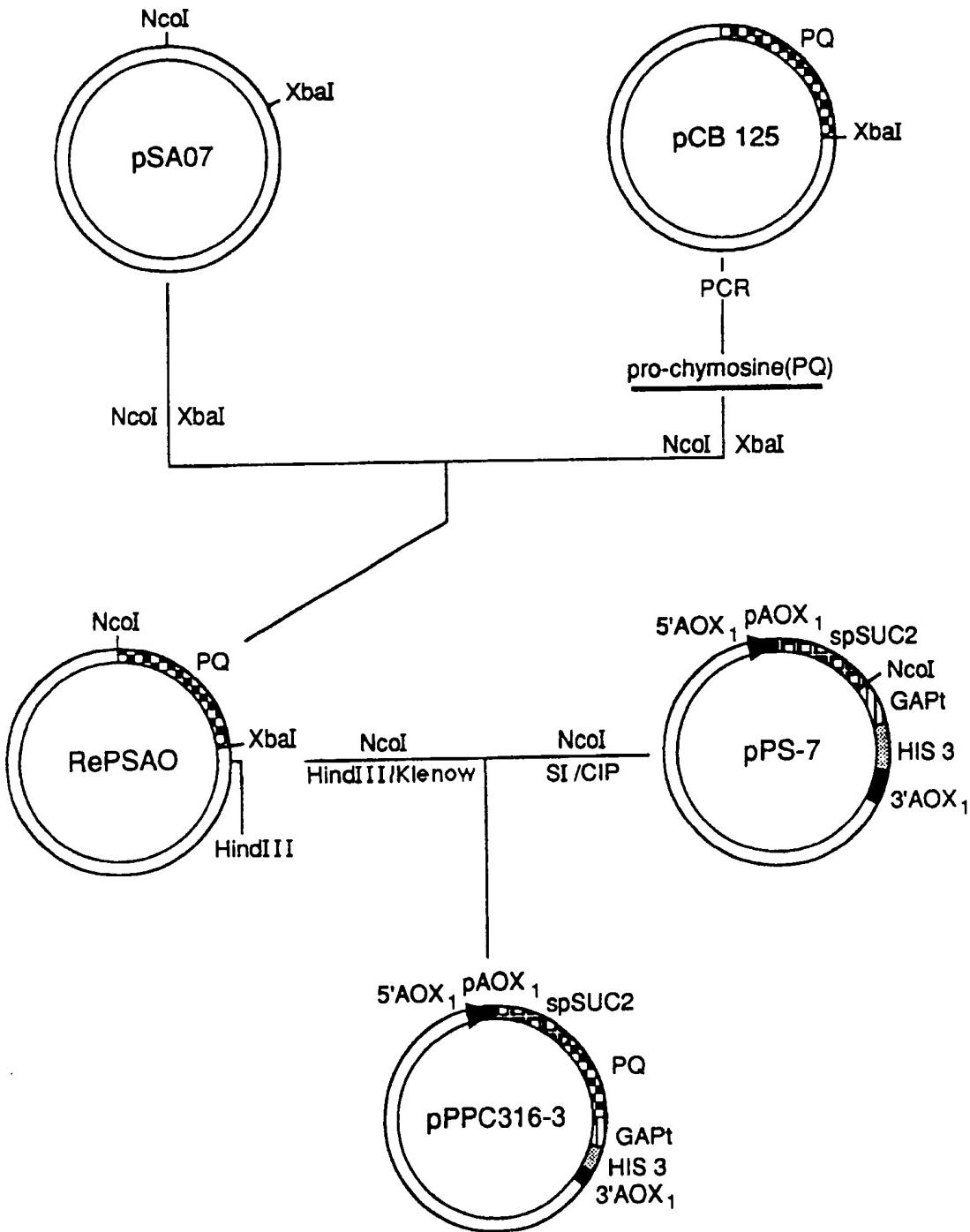


FIG. 7

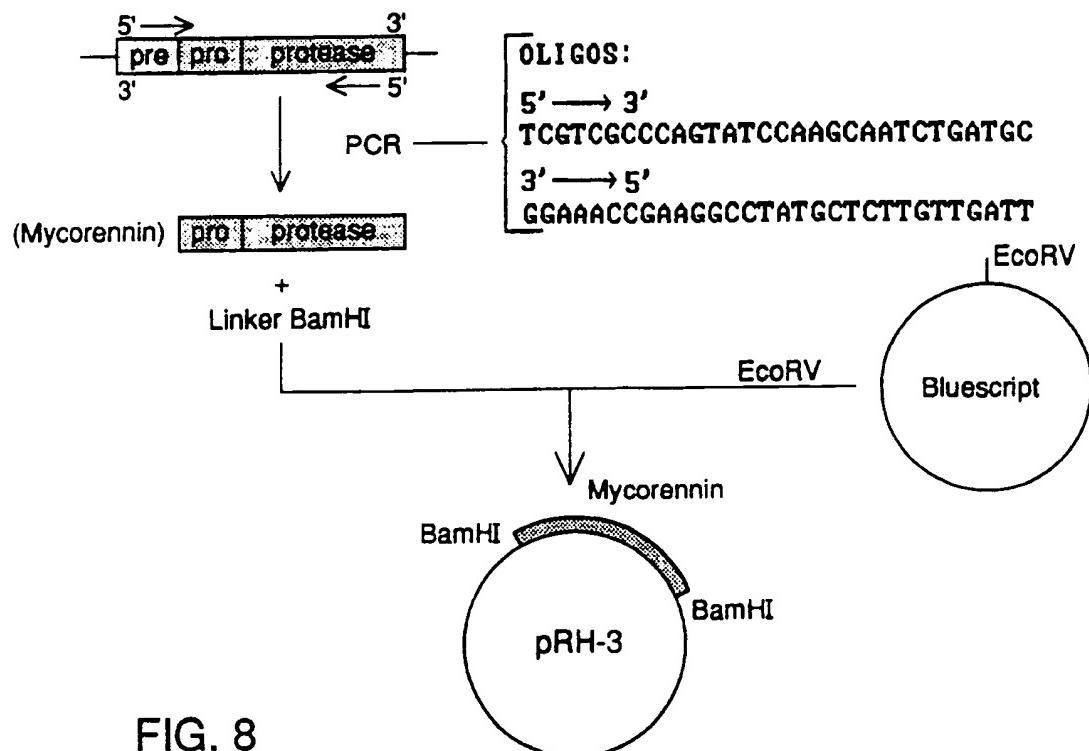


FIG. 8

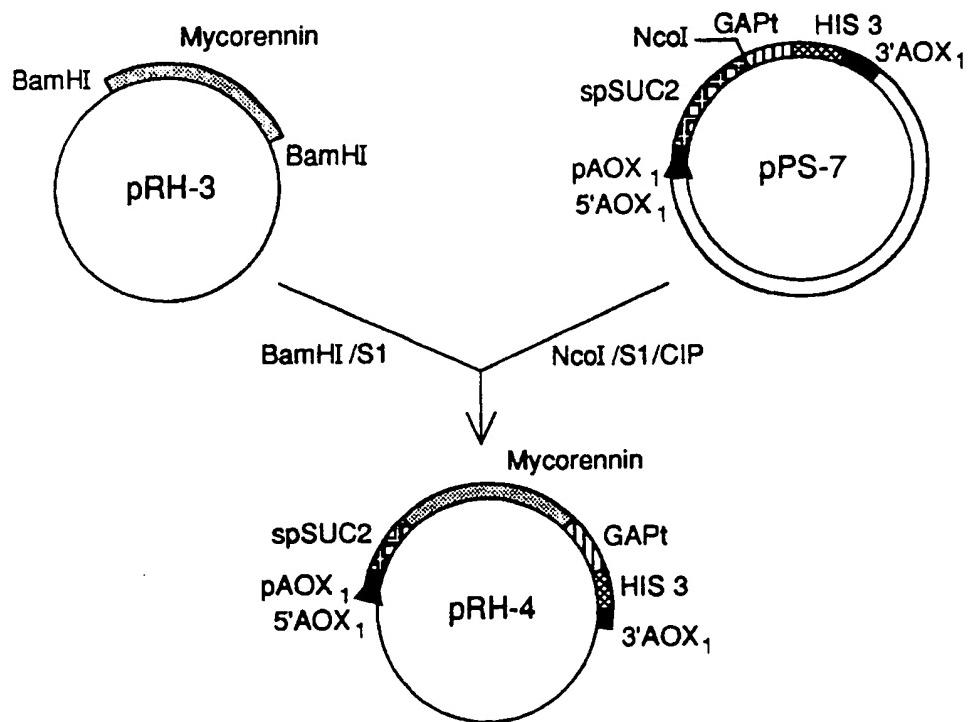


FIG. 9

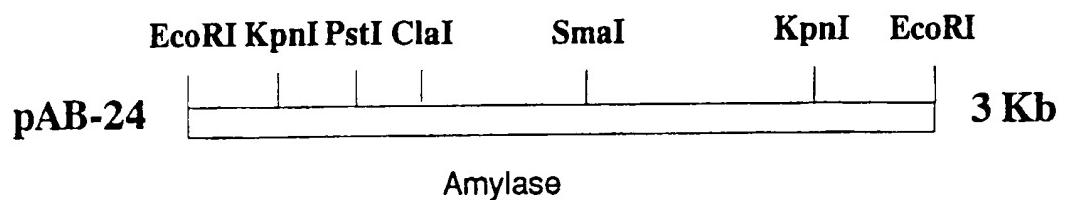


FIG. 10

5' - GCC ATG GCA AAT CTT AAT GGG A - 3'
3' - GT TAA ATA CAA GTT TCT ATC CAG CTG C - 5'

FIG. 11

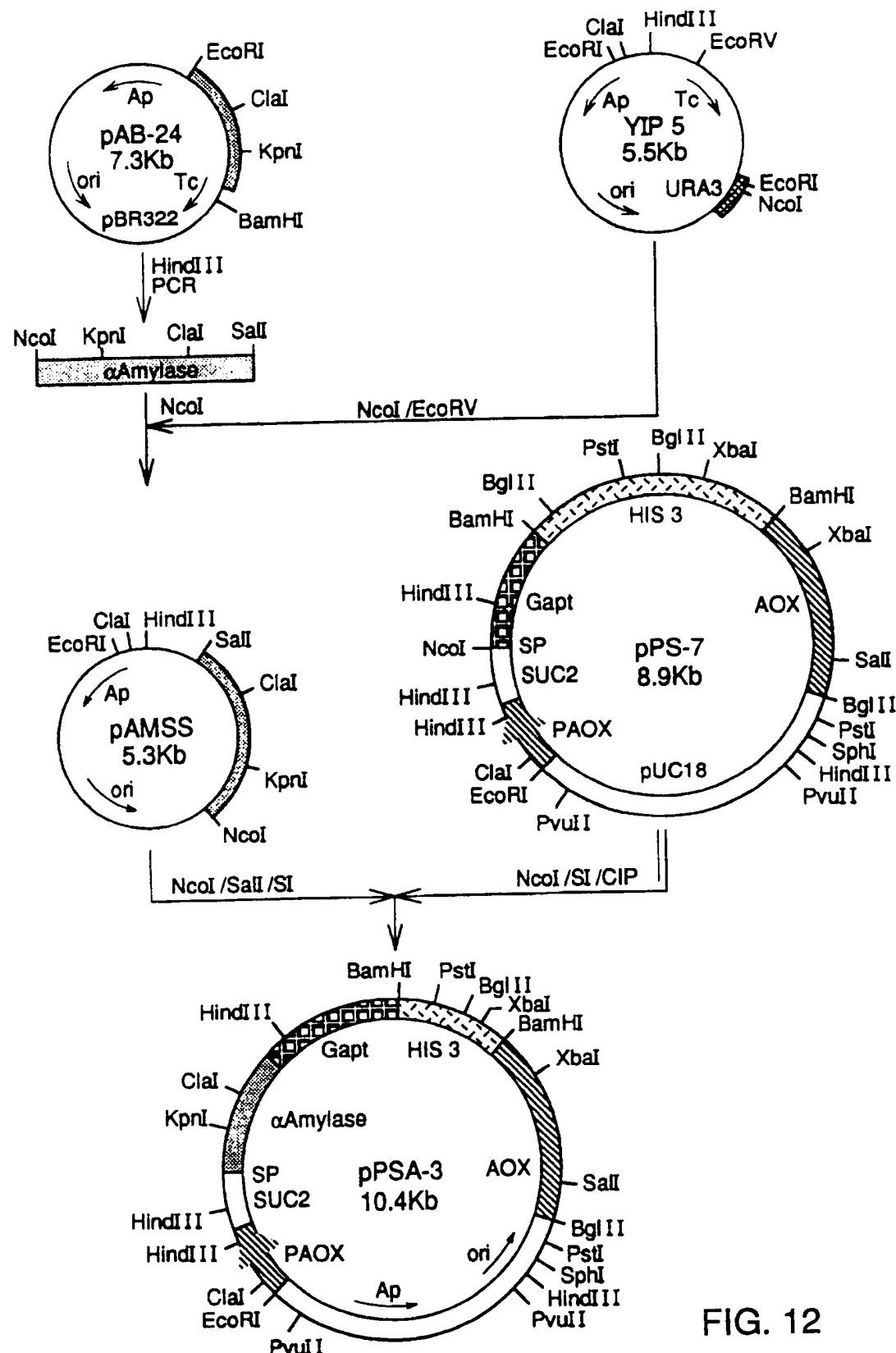


FIG. 12



EUROPEAN SEARCH
REPORT

Application Number

EP 91 20 0074

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl.5)
Y	BIOTECHNOLOGY vol. 5, no. 12, December 1987, NEW YORK US pages 1305 - 1308; J.F.Tschopp et al.: "High-level secretion of glycosylated invertase in the methylotrophic yeast, <i>Pichia pastoris</i> " * the whole document * - - -	1,9,12,14	C 12 N 15/81 C 12 N 15/16 C 12 N 15/57 C 12 N 15/56 C 12 N 1/16
Y	EP-A-0 263 311 (PHILLIPS PETROLEUM COMPANY) * page 10, line 13 - page 10, line 31; claims 1-10 ** page 12, lines 21 - 55 * - - -	1,9,12,14	
Y,A	EP-A-0 324 258 (GENEBIOMED, INC.) * claims 6, 7; figure 4 * - - -	1,9,12,14, 2,7	
D,A	GENE. vol. 23, no. 3, September 1983, AMSTERDAM NL pages 267 - 276; Ortlepp et al.: "Molecular cloning in <i>Bacillus subtilis</i> of a <i>Bacillus licheniformis</i> gene encoding a thermostable alpha amylase" * abstract * - - -	4,10,13, 14	
A,D,A	WO-A-8 500 369 (CHIRON CORPORATION) * claims 1, 3 & US-A-4783412 * - - -	4,10,13, 14	TECHNICAL FIELDS SEARCHED (Int. Cl.5)
A	EP-A-0 261 534 (MILES LABORATORIES ,INC.) * claims 1-3 * - - -	1,7,9	C 12 N C 12 R
A	EP-A-0 137 710 (GENEX CORPORATION) * page 5, line 20 - page 6, line 11 * - - -	4,10,13, 14	
A	CHEMICAL ABSTRACTS, vol. 108, 1988 Columbus, Ohio, USA Yamashita Takashi et al.: "Secretion of Mucor rennin, a fungal aspartic protease of Mucor & MGG, Mol. Gen. Genet. vol.210,1987,No.3,pp.462-7 ref. no. 217200Y * abstract * - - - -/-	4,10,13, 14	

The present search report has been drawn up for all claims

Place of search	Date of completion of search	Examiner
Berlin	10 April 91	GURDJIAN D P M

CATEGORY OF CITED DOCUMENTS

- X: particularly relevant if taken alone
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L: document cited for other reasons

&: member of the same patent family, corresponding document

EUROPEAN SEARCH
REPORT

Application Number

EP 91 20 0074

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl.5)
A	EP-A-0 329 127 (GREEN CROSS CORPORATION) * page 5, lines 7 - 8 *	1,3,5, 7-11	

TECHNICAL FIELDS SEARCHED (Int. Cl.5)			

The present search report has been drawn up for all claims			
Place of search	Date of completion of search	Examiner	
Berlin	10 April 91	GURDJIAN D P M	
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E: earlier patent document, but published on, or after the filing date D: document cited in the application L: document cited for other reasons &: member of the same patent family, corresponding document			